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13. ABSTRACT (Maximum 200 Words)

The primary accomplishments of the previous funding period were: 1) The 4.0 kb genomic sequence (-4000 to +1) immediately upstream of the transcriptional start site of the Scn10a gene was fused to the enhanced green fluorescence protein (EGFP) and microinjected into the nuclei of neurons of dorsal root ganglia (DRG). The -4000 to -2500 region was found to be essential for expression of EGFP; 2) The transcription factor c-Jun was found to bind within the -3100 to -3200 region; 3) At least 5 other transcription factors bind within the -3100 to -3200 region; 4) A large number of putative cDNAs encoding the binding domains of putative transcription factors that interact within the -3100 to -3200 region was isolated using the yeast one-hybrid technique; 5) A large collection of cDNAs encoding wild-type and mutant forms of Gα, Gβ, and Gγ subunits were constructed for future analysis into their role in activating the Scn10a tetrodotoxin-resistant sodium channel.

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Introduction

The Scn10a gene product encodes a tetrodotoxin-resistant sodium channel (SNS/PN3) expressed exclusively in a subset of primary sensory neurons (e.g., dorsal root and nodose ganglia) believed to be involved in pain transmission. Thus, it is important to understand mechanisms contributing to both the function of the protein and the exquisite specificity of gene expression. The overall research plan is detained in the flowchart depicted to the right. Significant progress was made during the latest funding period on both the genomic (left branch) and proteomic (right branch) sections of the research plan (figure 1).

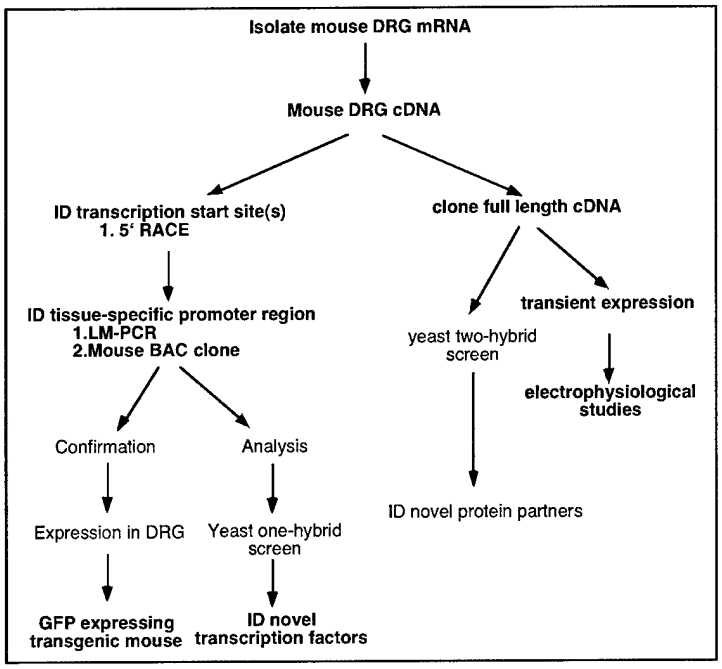

Figure 1. Research Plan.

Expression analysis of the 4.0 kb genomic sequence immediately upstream of the transcriptional start site of the Scn10a gene revealed that the 1.5 kb sequence distal to the transcriptional start site is essential for gene expression. The transcription factor c-Jun (AP1) was found to bind to the far upstream region of this 4.0 kb sequence. Evidence of additional, but as yet uncharacterized, transcription factor interaction in the far upstream region was detected. To isolate transcription factors that interact within sub-regions of the 4.0 kb region, we have utilized the yeast one-hybrid technology. A number of putative transcription factors that interact within specific regions essential for Scn10a gene transcription have been isolated and their identity is currently being assessed. Lastly, a wide range of cDNAs encoding wild-type and mutant (constitutively active or dominant negative) forms of Gα, Gβ, and Gγ subunits were constructed to assess their role in modulating expression of the Scn10a sodium channel in future co-transfection assays of mouse dorsal root ganglia (DRG).

Expression studies with 4.0kb region.

The sequence of the 4.0 kb region upstream of the transcriptional start site of the Scn10a gene is shown in figure 2.

Figure 2

Sequence Range: 1 to 4032

>LMPCR_(round_two)_product_SSP1_library

```
            |
            |       10        20        30        40        50        60
           ATTCCAGTTGCTGAGTGGAGAGAGCACTGTAGGGTCATGGAAGGACAGTGGGGAGGTCTG

                   70        80        90       100       110       120
           TTAGAGGTCCTTGAAATTATATAGTGACCTCGCCATGATGGTGGTCTCAGAGATCGAGAG

                  130       140       150       160       170       180
           ATGATGTAATCAGGAGGACTCTAGGAATTCAAGTTAGAGGCCCCAGAAAGAGGGCTGTGG

                  190       200       210       220       230       240
           ACGAGGGACGGCTCTTGGATTACCTCTAGATGCTGGGCTTGTGAGTCCAGGCAAGCAGAG

                  250       260       270       280       290       300
           TGTTCTTGGAGAGGCTTCTCTGGGGGAGGATCATTCTGAGCAGGGCACAGGCACAGAAAT

                  310       320       330       340       350       360
           CATTAGTCCATCTGTAAACATGTCTGAGATGTTAGTGGAGTGTCCATGAAGGGAAATTCA

                  370       380       390       400       410       420
           GGCTTCTACCACATTAGTGTATATTTAAATCTGACACCAGGAGAGAGATTTATGATGGAG

                  430       440       450       460       470       480
           CTGACAGACTCCGGTGCCATGTCAGGTAGGTGACTGAAGCCCTGGGGAAGGAGAGGCGTA

                  490       500       510       520       530       540
           GGATGGAATCTTAAAACGATTCTCCAACTACTTCCAGGTGGCAGAGGAGGAGGCAGCCCA

                  550       560       570       580       590       600
           GGCCAGAGAAGCTCCTCTGAAAACAGAAGTCAAGAGGGTGGAGTGTGGTGCAAGGACCAT

                  610       620       630       640       650       660
           GCAGCTAATCCTGCGGAGCCCCTAGGATGAGAGCGCCAGAGAGGAGACACATGACACAGG

                  670       680       690       700       710       720
           GAGACCAGTAGAAACCTGTTAAGATTCCGGGTGTCTCAGGACTGCCTCTGGATGCACACT

                  730       740       750       760       770       780
           TCTTCCTTCTTGGGAAGTTACTTTTCTGTCACTGTGATGAAATACCTTAACCAAGGTGAC

                  790       800       810       820       830       840
           TCAAAGAAGAGAGGGTTTATCTGGGCTCACGGGTCCAGAGGTAGAGGAACACATGGAGAT

                  850       860       870       880       890       900
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                  910       920       930       940       950       960
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                  970       980       990      1000      1010      1020
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                 1030      1040      1050      1060      1070      1080
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      1150       1160       1170       1180       1190       1200
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      1270       1280       1290       1300       1310       1320
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      1330       1340       1350       1360       1370       1380
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      1390       1400       1410       1420       1430       1440
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                          >overlap_LMPCR_round_1+2
                                      |
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>LMPCR_(round_one)_product_EcoRV_library
                |
      1630       1640       1650       1660       1670       1680
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      1750       1760       1770       1780       1790       1800
CATGACATTTTTAGTAAATTCTTTGAATTTTTTTGAGACAGGGTCTTACACTACAGCTCA

      1810       1820       1830       1840       1850       1860
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      1870       1880       1890       1900       1910       1920
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      1930       1940       1950       1960       1970       1980
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      1990       2000       2010       2020       2030       2040
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      2110       2120       2130       2140       2150       2160
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      2170       2180       2190       2200       2210       2220
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```

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2470 2480 2490 2500 2510 2520
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2530 2540 2550 2560 2570 2580
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2770 2780 2790 2800 2810 2820
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2830 2840 2850 2860 2870 2880
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2950 2960 2970 2980 2990 3000
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3130 3140 3150 3160 3170 3180
GCATGGGCCCTCGACAGTTTCCTGCAGTATAGCATGGCTTCCTAAGGCTGCGTGGGTTGC

3190 3200 3210 3220 3230 3240
ACTGTTACGGAGGGCTCAGCTCAGACAGGGGGTTCCCTGTGCAACCTCCTTTCTTATGGT

3250 3260 3270 3280 3290 3300
CCCACAACCCCACAGATAGGGCACTTTCCCTACCCAGCTCCCTTCTCGGCTCTCACTGGG

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3370 3380 3390 3400 3410 3420
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3430 3440 3450 3460 3470 3480
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3490 3500 3510 3520 3530 3540
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3550 3560 3570 3580 3590 3600

```
CAACGGGTGCTGCTCTGCCACGCAGGGGCAGCGGTGGGACTCAGCCCATCCTGCTAAGGA

      3610       3620       3630       3640       3650       3660
CGGGCAGCCTGAGCCAGGCTTGGGAGTCTGTCATGGCTGCCAGACGAATCATTATCTAAT

      3670       3680       3690       3700       3710       3720
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      3730       3740       3750       3760       3770       3780
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      3790       3800       3810       3820       3830       3840
TCAGAGTGTACTTTCTGGAGCCCATCCAGCAAGCAGGGTGGAACTCATGACGGGAAATGG

      3850       3860       3870       3880       3890       3900
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                                >RACE_clone_B_end
                                        |
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           >RACE_clone_I_end
                    |
      3970       3980       3990       4000       4010       4020
AGCTGGGGTCTCCAGCTTACTTCTGCTAATGCTACCCCAGGCCTTTAGACGGAGAACAGA

      4030
TGGCAGATGGAG
```
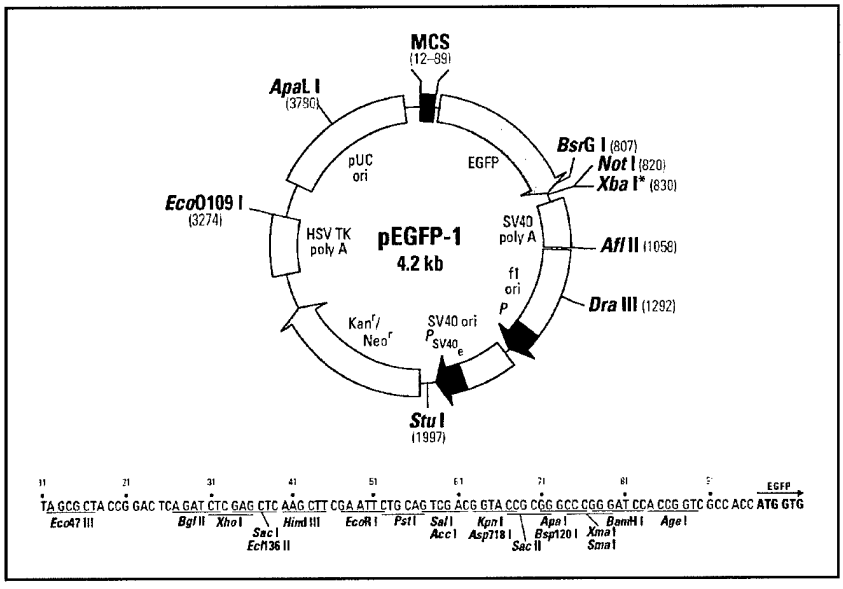

Two PCR products, 1.7 kb and 2.5 kb in size, corresponding to the transcriptional start site distal and proximal portions of the 4.0 kb sequence were cloned into the pEGFP-1 vector from Clontech (figure 2). This vector contains the coding region of the enhanced green fluorescent protein down stream from a multiple cloning site. The vector allows the analysis of sequences for promoter activity by their ability to drive expression of the EGFP protein product.

Figure 2. pEGFP expression vector.



The resulting expression constructs were microinjected into the nuclei of neurons from primary cultures of dorsal root ganglia. A nuclear targeted dsRED construct was coinjected as a positive control. The presence of visibly red nuclei indicated a successful injection yet would not interfere with the detection of the EGFP signal that was predominantly cytoplasmic. The neurons were dissociated with collagenase and trypsin and cultured for two days in the presence of nerve growth factor and glial derived neurotrophic factor. The construct containing the 2.5kb fragment failed to produce visible EGFP production as shown in figure 3. The 4.0kb fragment successfully drove expression in a majority of but not all injected cells.

Figure 3. The 2.5 kb fragment does not drive expression of EGFP in mouse DRG neurons.

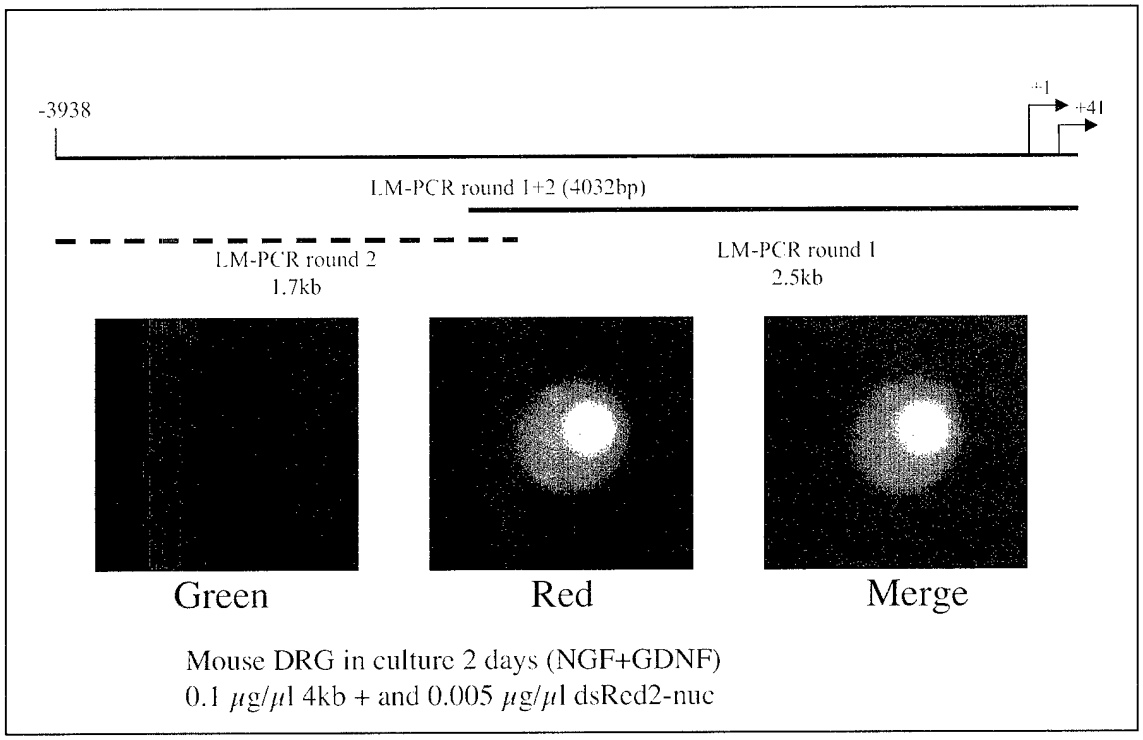
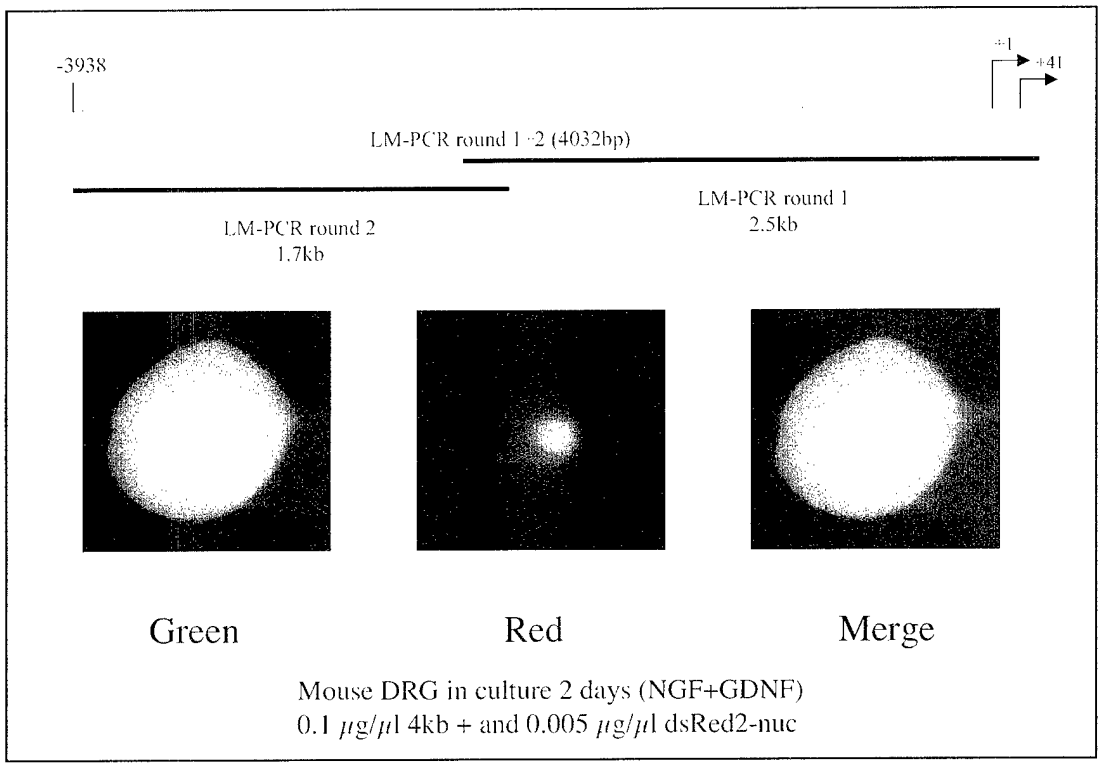


Figure 4 shows cells expressing EGFP following injection. Figure 5 shows an example of a successful injection, as viewed by dsRED production, with no EGFP production. The expression of Scn10a in only a subset of small diameter neurons in DRGs may account for the failure of this construct to express in all injected cells. Injection of all constructs into sympathetic neurons isolated from superior cervical ganglia failed to produce visually detectable levels of EGFP. Scn10a is not expressed in these neurons and therefore this experiment serves as a negative control.

Figure 4. The 4.0 kb construct drives expression of EGFP in DRGs



Deletion analyses of the 4.0kb fragment has begun. Three deletion fragments of the 5' end of the 4.0kb fragment, designated S, M, and L, have been generated by the PCR and cloned into pEGFP-1. These constructs designated S, M, and L were generated by designing primers to various positions of the parent 4.0kb fragment as shown in figure 5. Preliminary injection experiments performed as described above with the M or medium sized construct produced visibly green cells. This suggests that essential *cis* elements lie within the region in construct M. Sequence analysis of the 4.0 kb region (figure 6 and 7) reveals a number of putative *cis* elements and silencer elements that bind specific transcription factors.

Figure 5. 5' Deletion strategy for 4.0kb deletion constructs.

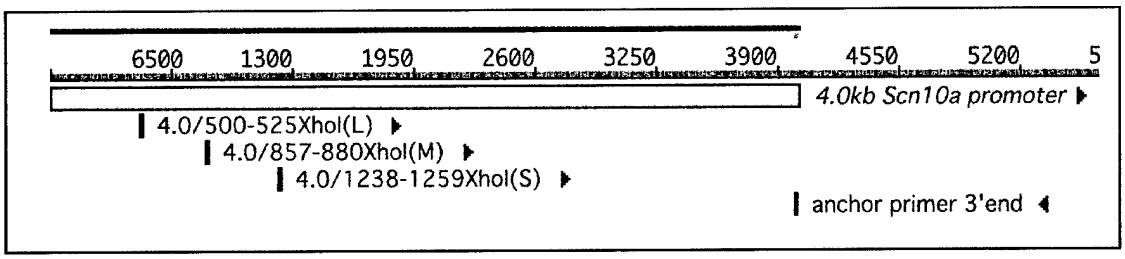


Figure 6. Putative *cis* elements in the 4.0 kb region.

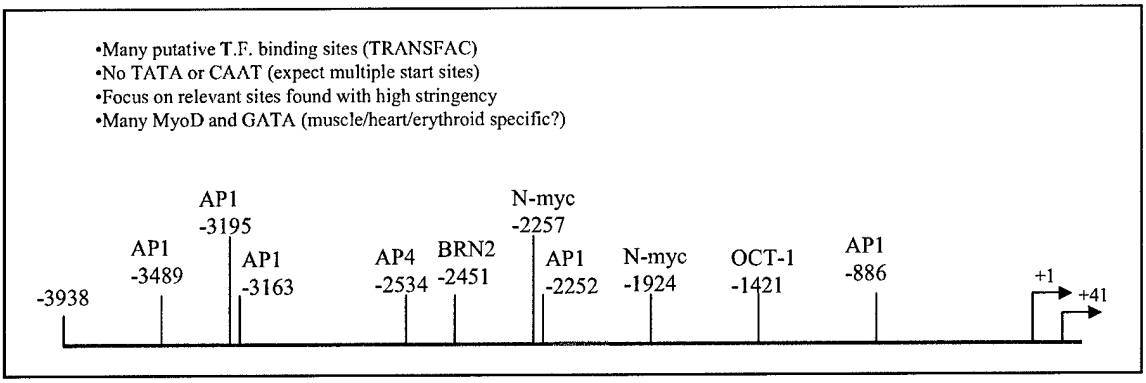
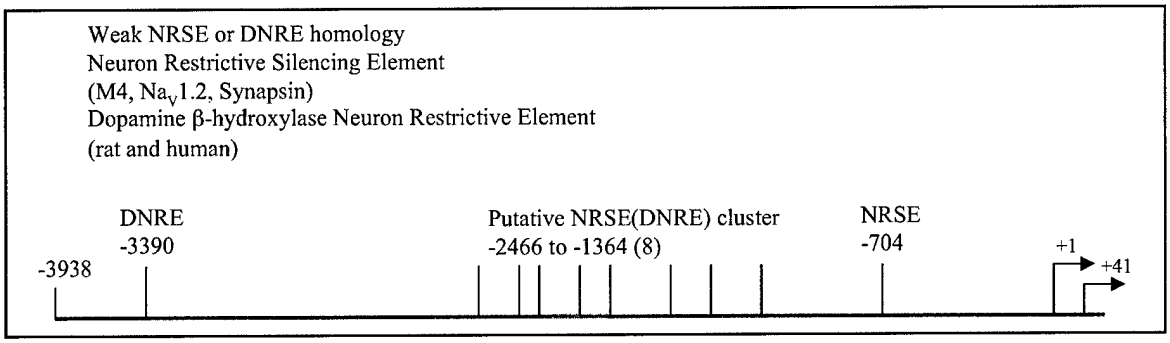


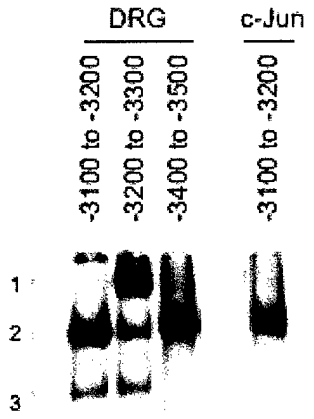
Figure7. Location of putativesilencer elements NRSE (Neuron restrictive silencer element) and DNRE (Dopamine beta-hydroxylase neuron restrictive element).



Electrophoretic mobility shift analysis (EMSA) of the 4.0 kb region.

Since the 2.5 kb fragment was not able to drive expression of EGFP in transfected DRGs but the 4.0 kb region could not, this indicates that the 1.5 kb region distal to the transcriptional start site contained essential *cis* elements. Therefore, the focus of this granting period was on identifying essential *cis* elements in the 1.5 kb region. The 1.5 kb region was divided into 100 bp sections (15 total) by the PCR and each 100 bp fragment was labeled with [γ^{32}P]ATP and incubated with nuclear extract protein from DRGs. Three regions, -3100 to –3200, -3300 to –3400, and -3400 to –3500, were able to bind one or more proteins present in the DRG nuclear extract (figure 8). Analysis of the sequences from these regions (figures 6 and 7) indicates the presence of putative binding sites for the AP1 protein, c-Jun, and a neuron restrictive silencer element (NRSE). When purifed c-Jun was incubated with each sub-region, binding of c-Jun to the –3100 to –3200 was evident (figure 8, lane 4). Therefore, one of the two DNA/protein complexes visualized when the –3100 to –3200 region was incubated with DRG nuclear extract protein contained c-Jun protein. The identities of the other nuclear extract proteins bound to the three regions are presently unknown.

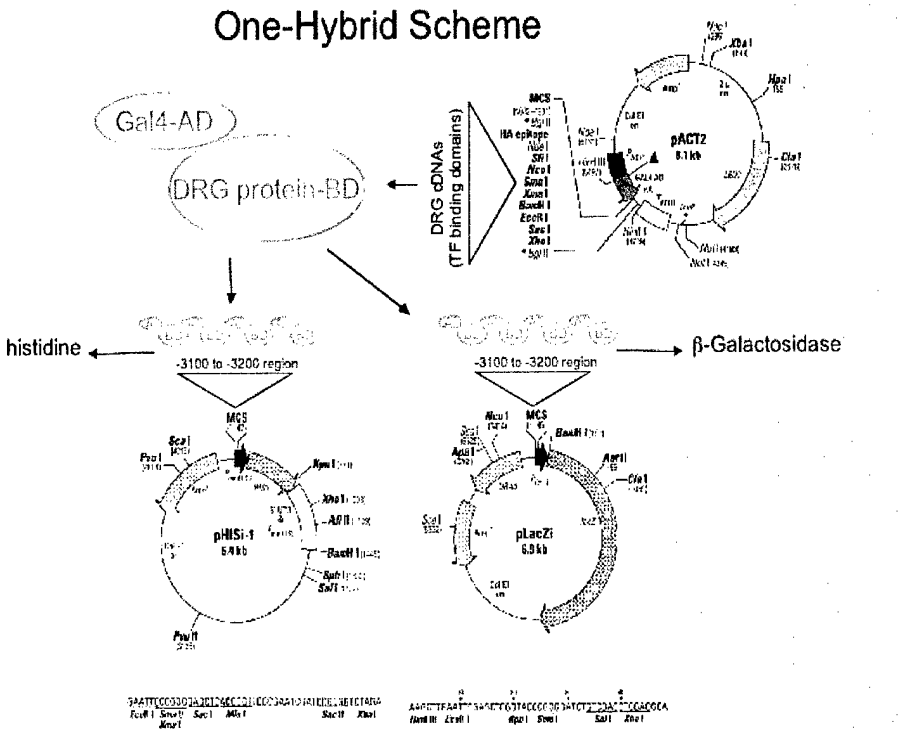
Figure 8. EMSA of specific sub-regions of the 4.0 kb region.



One-hybrid analysis to isolate transcription factors bound to the 4.0 kb region.

The yeast one-hybrid technique is currently in use to identify transcription factors from DRGs that bind to the three sub-regions of the 4.0 kb region. The general scheme for one-hybrid screening is shown in figure 9.

Figure 9. One-hybrid analysis of the –3100 to –3200 region.



Transcription factors contain at least two domains, a binding domain (BD) and an activation domain (AD). The BD is used to bind specific DNA sequences within the promoter for a gene, whereas the AD is needed for the transcription factor to activate gene transcription. The one-hybrid analysis uses the 100 bp sub-regions from the 4.0 kb region as a target for any protein containing a BD for a specific DNA sequence on each sub-region. The AD is supplied by the Gal4 transcription factor. A cDNA library was constructed from total RNA isolated from mouse DRGs and ligated into the vector pACT2 (Clontech) that contains the AD for Gal4 upstream of a multiple cloning site for the DRG cDNAs. Target reporter genes containing each 100 bp sub-region fused upstream of the yeast gene *HIS3* were constructed in the vector pHISi-1 (Clontech). When the DRG-pACT2 library is transformed into yeast cells, the DRG cDNAs are expressed as fusion proteins to the Gal4-AD. If the DRG-pACT2 library is transformed into a yeast strain containing an integrated copy of each 100 bp-pHISi-1 vector, any fusion protein containing a BD for specific DNA sequences in the 100 bp sub-regions is expected to bind within the 100 bp region and activate gene transcription of the *HIS3* gene. Growth of a histidine-requiring yeast strain containing an integrated copy of a 100 bp-pHISi-1 vector on media lacking histidine indicates that a fusion protein capable of binding to the 100 bp sub-region is present in the transformed strain. Using this approach, we identified 42 DRG-pACT2 clones that contain putative BDs for DRG transcription factors that bind to the –3100 to –3200 sub-region of the Scn10a promoter. We currently are analyzing these clones by DNA sequence analysis to determine whether

they correspond to known transcription factors or are novel. As time permits, the other two sub-regions will be analyzed in the same fashion.

Cloning wild-type and mutant Gα, Gβ, and Gγ subunits for future analysis of their effect on Scn10a function.

The purpose of these experiments is to determine whether G protein α and/or βγ subunits modulate the Scn10a sodium channel in sensory neurons. Various combinations of G proteins will be co-expressed in mouse DRG or nodose ganglion neurons, and whole-cell voltage-clamp recording of tetrodotoxin-resistant sodium channel activity will be made using the patch-clamp technique. During this funding cycle, a large number of wild-type, constitutively active, and dominant negative forms of Gα, Gβ, and Gγ genes have been isolated by our Guthrie cDNA Resource Center staff (see website www.cdna.org). The number of clones has expanded greatly since the start of this proposal. The cDNAs were prepared by the PCR using DNA primers specific to known G proteins and subcloned into two mammalian expression vectors, pcDNA 3.1 (InVitrogen) and PDNR-1r (Clontech). The clones were sequence-verified, and expression verified in most cases by coupled *in vitro* transcription/translation assays and the catalog of clones is shown (appendix A).

Cloning of the wild-type G-protein αolf subunit (appendix B) and its constitutively active form (Appendix C) is given as an example of the clones isolated by the Guthrie cDNA Resource Center. The complete coding sequence for wild-type αolf and the location of the mutation introduced to change a glutamine (Q) to leucine (L) to eliminate GTPase activity yielding a constitutively active phenotype is indicated.

Key Research Accomplishments

1. Analysis of the 4.0 kb genomic sequence immediately upstream of the transcriptional start site of the Scn10a gene revealed that the distal 1.5 kb portion was essential for gene activation in DRGs.
2. The transcription factor c-Jun was shown to bind *in vitro* within the –3100 to –3200 region contained on this 4.0 kb fragment.
3. At least five other transcription factors bind within the region –3100 to –3500, and their identities are as yet unknown.
4. A large collection of cDNAs containing binding domains for putative transcription factors that interact within the –3100 to –3200 region were identified by a yeast one-hybrid protocol.
5. A large collection of cDNAs encoding wild-type and mutant forms of Gα, Gβ, and Gγ subunits were constructed for future analysis into their role in activating the Scn10a tetrodotoxin-resistant sodium channel.

Reportable Outcomes

None

Conclusions

The focus of this funding period has been on the 4.0 kb genomic fragment immediately upstream of the transcriptional start site for the Scn10a gene. The distal 1.5 kb portion was shown to be essential for Scn10a gene expression in transfected DRGs. Because of the relatively large size of this region, we sub-divided it into 100 bp sections and analyzed these regions by EMSA for binding of DRG nuclear extract protein and found that the –3100 to –3500 region efficiently bound several proteins *in vitro*. The DNA sequence of this region showed the presence of AP1 (c-Jun) binding sites that was confirmed by EMSA with purified c-Jun protein.

To date, the –3100 to –3200 region has been analyzed for transcription factor binding sites using the yeast one-hybrid assay. We have isolated 42 cDNA clones that contain at least the binding domains for putative transcription factors that interact within this region. Analysis of these cDNAs is in progress including isolation of their full-length coding sequence. This will allow us to determine whether any of these cDNAs are functional in co-transfection analyses along with Scn10a promoter-EGFP reporter constructs into primary cultures of DRGs. The –3200 to –3500 region is to be included in the focus of the next funding cycle.

We have cloned a large number of cDNAs, both wild-type and mutant forms, for Gα, Gβ, and Gγ subunits that are expected to be useful in future experiments to determine their role in regulating expression of the tetrodotoxin-resistant Scn10a sodium channel.

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Appendix A

Guthrie cDNA Resource Center Clone Catalog, November, 2002 (485 clones)

www.cdna.org

HETEROTRIMERIC G PROTEINS

α	α							β	β		γ	γ		RGS	RGS		GPR GoLoco
WT	WT	WT/EE	QL	QL/EE	XTP	PTX-R	GT	WT	WT	FLAG	WT	WT	HA	WT	WT	3X-HA	WT
α cone	α cone	α cone	α cone	α cone	α cone	C→I	α i1	β 1	β 1	β 1	γ 1	γ 1	γ 1	RGS1	RGS1	RGS1	rAGS3S
α rod	α rod	α rod	α rod	α rod	α rod	C→S	α i2	β 2	β 2	β 2	γ 2	γ 2	γ 2	RGS2	RGS2	RGS2	rAGS3L
α i1	α i1	α i1	α i1	α i1	α i1	α rod	α i3	β 3	β 3	β 3	γ 3	γ 3	γ 3	RGS3	RGS3	RGS3	LGN
α i2	α i2	α i2	αi2	α i2	α i2	α i1	α oA	β 3S	β 3S	β 3S	γ 4	γ 4	γ 4	RGS3T	RGS3T	RGS3T	
α i3	α i3	α i3	α i3	α i3	α i3	α i2	α oB	β 4	β 4	β 4	γ 5	γ 5	γ 5	RGS4	RGS4	RGS4	
	α olf	α olf	α olf	α olf	α olf	α i3		mβ 4	mβ 4		γ 7	γ 7	γ 7	RGS5	RGS5	RGS5	
α sS	α sS	α sS	α sS	α sS	α sS	α oA			β 5	β 5	γ 8	γ 8	γ 8	RGS7(S1)	RGS7(S1)	RGS7(S1)	
α sL	α sL	α sL	α sL	α sL	α sL	α oB		β 5L	β 5L	β 5L	γ 9	γ 9	γ 9		RGS7(S2)	RGS7(S2)	
α q	α q	α q	α q	α q	α q	C→G					γ 10	γ 10	γ 10	RGS8	RGS8	RGS8	
α 11	α 11	α 11	α 11	α 11	α 11	α cone					γ 11	γ 11	γ 11	RGS9	RGS9	GAIP	
α12	α12	α 12	α 12	α 12	α 12	α rod					γ 12	γ 12	γ 12	RGS9L	RGS9L	RGS10	
α 13	α 13	α 13	α 13	α 13	α 13	α i1					γ 13	γ 13	γ 13	GAIP	GAIP	RGS13	
α 14	α 14	α 14	α 14	α 14	α 14	α i2								RGS10	RGS10	RGS14S	
α 15(16)	α 15(16)	α 15(16)	α 15(16)	α 15(16)	α 15(16)	α i3								RGS13	RGS13	RGS16	
α z	α z	α z	α z	α z	α z	α oA								RGS14S	RGS14S	RGS17	
α oA	α oA	α oA	α oA	α oA	α oA	α oB								RGS16	RGS16	RGSZ1	
αoB	αoB	α oB	α oB	α oB	α oB									RGS17	RGS17		
														RGSZ1	RGSZ1		

RAS	RAS						Rho	Rho						Rab	Rab	ARL	ARL	GPCR		ION-Ch
WT	WT	3X-HA/WT	GV	3X-HA/GV	SN	3X-HA/SN	WT	WT	3X-HA/WT	GV	3X-HA/GV	TN	3X-HA/TN	WT	WT	WT	WT	WT	3X-HA	WT
H-Ras	H-Ras	H-Ras	H-Ras	H-Ras	H-Ras	H-Ras	Cdc42	Cdc42	Cdc42	Cdc42	Cdc42	Cdc42	Cdc42	Rab1A	Rab1A	Arf1	Arf1	M1	M1	HTR3A
N-Ras	N-Ras	N-Ras	N-Ras	N-Ras	N-Ras	N-Ras	Cdc42p	Cdc42p	Cdc42p	Cdc42p	Cdc42p	Cdc42p	Cdc42p	Rab2A	Rab2A		Arf3	M2	M2	
K-Ras	K-Ras	K-Ras	K-Ras	K-Ras	K-Ras	K-Ras	RhoA	RhoA	RhoA	RhoA	RhoA	RhoA	RhoA	Rab3A	Rab3A	Arf4	Arf4	M3	M3	
M-Ras	M-Ras	M-Ras	M-Ras	M-Ras	M-Ras	M-Ras	RhoB	RhoB	RhoB	RhoB	RhoB	RhoB	RhoB	Rab3B	Rab3B	Arf5	Arf5	M4	M4	
R-Ras	R-Ras	R-Ras	R-Ras	R-Ras	R-Ras	R-Ras	RhoC	RhoC	RhoC	RhoC	RhoC	RhoC	RhoC	Rab3D	Rab3D	Arf6	Arf6	M5	M5	
Rap1A	Rap1A						Rac1	Rac1	Rac1	Rac1	Rac1	Rac1	Rac1	Rab4A	Rab4A		Arf Prot	κO		
Rap1B	Rap1B	**Ras-like**	**Ras-like**	**Ran**		**Adapters**	Rac2	Rac2	Rac2	Rac2	Rac2	Rac2	Rac2	Rab4B	Rab4B	Arl1	Arl1	ADORA1		
Rap2A	Rap2A	WT	WT	WT	3X-HA	WT	Rac3	Rac3	Rac3	Rac3	Rac3	Rac3	Rac3	Rab5A	Rab5A	Arl2	Arl2	ADORA2A		
Rap2B	Rap2B	AGS1	AGS1	Ran	Ran	Grb2	RhoD	RhoD						Rab5B	Rab5B	Arl3	Arl3	ADORA2B		
			Dex Ras2	RCC1	RCC1		RhoG	RhoG						Rab5C	Rab5C	Arl4	Arl4	ADORA3		
		RalA	RalA				RhoH	RhoH						Rab6A	Rab6A	Arl5	Arl5	ARB2		
			RalB				Rho6	Rho6						Rab6B	Rab6B	Arl7	Arl7	DRD1		
		Rheb2	Rheb2				Rho7	Rho7						Rab6C	Rab6C			DRD5		
			Rig				Rho8	Rho8						Rab7	Rab7			P2RY1		
		Rin	Rin				TCL	TCL						Rab8	Rab8			P2RY2		
		Rit	Rit				TC10	TC10						Rab9	Rab9			P2RY6		
			TC21											Rab10	Rab10			P2RY11		
														Rab11A	Rab11A			P2RY12		
														Rab11B	Rab11B			HRH1		
															Rab13			HRH2		
															Rab14			HRH4		
															Rab18			HTR1A		
														Rab23	Rab23			HTR1D		
														Rab26	Rab26			HTR1E		
														Rab27A	Rab27A			HTR1F		
															Rab27B			HTR2A		
															Rab28			HTR2B		
															Rab30			HTR2C		
															Rab31			HTR5A		
															Rab32					
															Rab33A					
															Rab35					

RhoGDI
WT
α
β
γ

genes cloned in the Creator™ donor vector, pDNR-1r

genes cloned in the pcDNA3.1+ vector

Phenotypes: **WT,** Wild type (i.e., native protein); **QL** constitutively-active due to a glutatmine (Q) to leucine (L) mutation which eliminates GTPase activity; **GV** constitutively-active due to a glycine (G) to valine (L) mutation which eliminates GTPase activity; **GT, SN, TN** dominant negative phenotypes due to glycine (G) to threonine (T), serine (S) to asparagine (N) or threonine (T) to asparagine (N) mutations, respectively; **PTX-R,** Pertussis toxin resistant due to mutation of a cysteine (C) to either isoleucine (I), glycine (G) or a serine (S), as indicated; **XTP,** dominant negative phenotype due to double glutamine (Q) to leucine (L) and aspartate (D) to asparagine (N) mutations;

Tags: **FLAG,** Epitope-tagged with the FLAG epitope; **EE,** Epitope-tagged with an internal glutamate-glutamate epitope; **HA,** Epitope-tagged with the hemagglutinin epitope.

GPCR, G protein coupled receptors; **M1-5,** muscarinic acetylcholine receptors; **kO** kappa opioid receptor; **DRD,** dopamine receptor; **HTR,** serotonin receptor; **P2R,** purinergic receptor; **ADORA,** adenosine receptor; **HRH,** histamine receptor; **RGS,** regulator of G protein signaling; **AR,** adrenergic receptor; **RhoGDI,** Rho guanine nucleotide dissociation inhibitor; **GPR,** G protein regulatory; **ION-Ch,** Ligand-gated ion channel.

Appendix B

Guthrie cDNA Resource Center
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1 Guthrie Square
Sayre, PA 18840

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email: cDNA@inet.guthrie.org
voice: (570) 882-4622
fax: (570) 882-4643

G-protein alpha olf

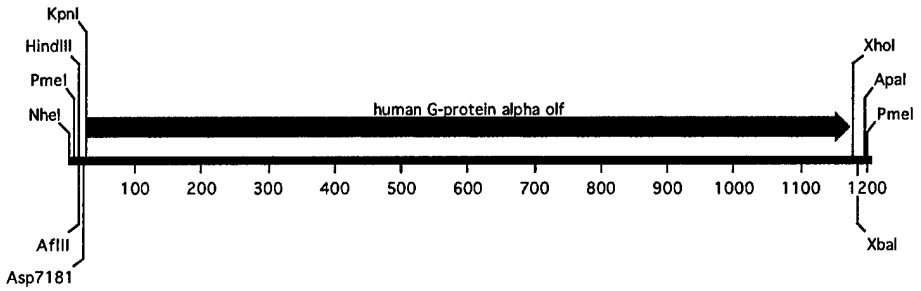
CloneID	GNA0L00000	**Species**	human
Gene Class	G-protein alpha	**IMAGE clone #**	
Date		**IMAGE acc. #**	
Lot	01	**Origin**	cDNA
Bacteria	JM109	**Tag**	None
Vector	pcDNA3.1+	**Tag location**	N/A
Antibiotic	Ampicillin	**Mutation**	None
Promoter	CMV	**Phenotype**	wt
Insert size	1150	**Method**	N/A
5'RE	KpnI	**Sequenced**	Full length
3'RE	XhoI	**GB Acc. No.**	U55184

Keywords guanine nucleotide binding protein alpha human wild-type

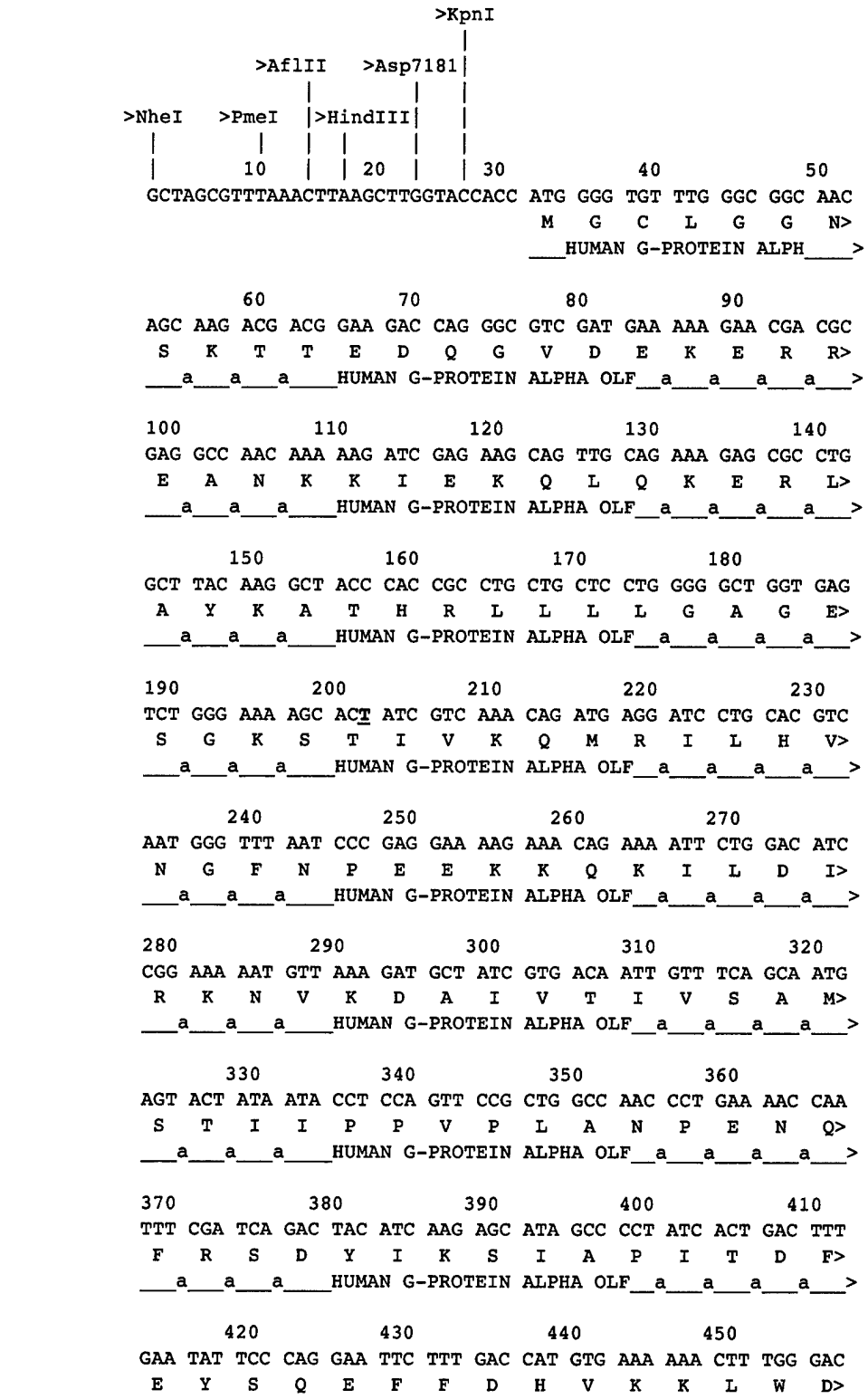
References

Notes Human G-protein alpha olf subunit (wild type) cloned into pcDNA3.1+ (Invitrogen) at KpnI (5') and Xho I (3'). The open reading frame was amplified by the PCR from human whole brain cDNA (Clontech). The insert was sequenced and found to be identical with GB ACC# U55184 with the following exceptions: nucleotide C171->T (silent). Insert size = 1150 bp.

Map



Human G-protein alpha olf



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 D   E   G   V   K   A   C   F   E   R   S   N   E   Y   Q>
___a___a___a____HUMAN G-PROTEIN ALPHA OLF__a___a___a___a___>

       510           520           530           540
CTG ATT GAC TGT GCA CAA TAC TTC CTG GAA AGA ATC GAC AGC GTC
 L   I   D   C   A   Q   Y   F   L   E   R   I   D   S   V>
___a___a___a____HUMAN G-PROTEIN ALPHA OLF__a___a___a___a___>

550           560           570           580           590
AGC TTG GTT GAC TAC ACA CCC ACA GAC CAG GAC CTC CTC AGA TGC
 S   L   V   D   Y   T   P   T   D   Q   D   L   L   R   C>
___a___a___a____HUMAN G-PROTEIN ALPHA OLF__a___a___a___a___>

       600           610           620           630
AGA GTT CTG ACA TCT GGG ATT TTT GAG ACA CGA TTC CAA GTG GAC
 R   V   L   T   S   G   I   F   E   T   R   F   Q   V   D>
___a___a___a____HUMAN G-PROTEIN ALPHA OLF__a___a___a___a___>

640           650           660           670           680
AAA GTA AAC TTC CAC ATG TTT GAT GTT GGT GGC CAG AGG GAT GAG
 K   V   N   F   H   M   F   D   V   G   G   Q   R   D   E>
___a___a___a____HUMAN G-PROTEIN ALPHA OLF__a___a___a___a___>

       690           700           710           720
AGG AGA AAA TGG ATC CAG TGC TTT AAC GAT GTC ACA GCT ATC ATT
 R   R   K   W   I   Q   C   F   N   D   V   T   A   I   I>
___a___a___a____HUMAN G-PROTEIN ALPHA OLF__a___a___a___a___>

730           740           750           760           770
TAC GTC GCA GCC TGC AGT AGC TAC AAC ATG GTG ATT CGA GAA GAT
 Y   V   A   A   C   S   S   Y   N   M   V   I   R   E   D>
___a___a___a____HUMAN G-PROTEIN ALPHA OLF__a___a___a___a___>

       780           790           800           810
AAC AAC ACC AAC AGG CTG AGA GAG TCC CTG GAT CTT TTT GAA AGC
 N   N   T   N   R   L   R   E   S   L   D   L   F   E   S>
___a___a___a____HUMAN G-PROTEIN ALPHA OLF__a___a___a___a___>

820           830           840           850           860
ATC TGG AAC AAC AGG TGG TTA CGG ACC ATT TCT ATC ATC TTG TTC
 I   W   N   N   R   W   L   R   T   I   S   I   I   L   F>
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       870           880           890           900
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 L   N   K   Q   D   M   L   A   E   K   V   L   A   G   K>
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 S   K   I   E   D   Y   F   P   E   Y   A   N   Y   T   V>
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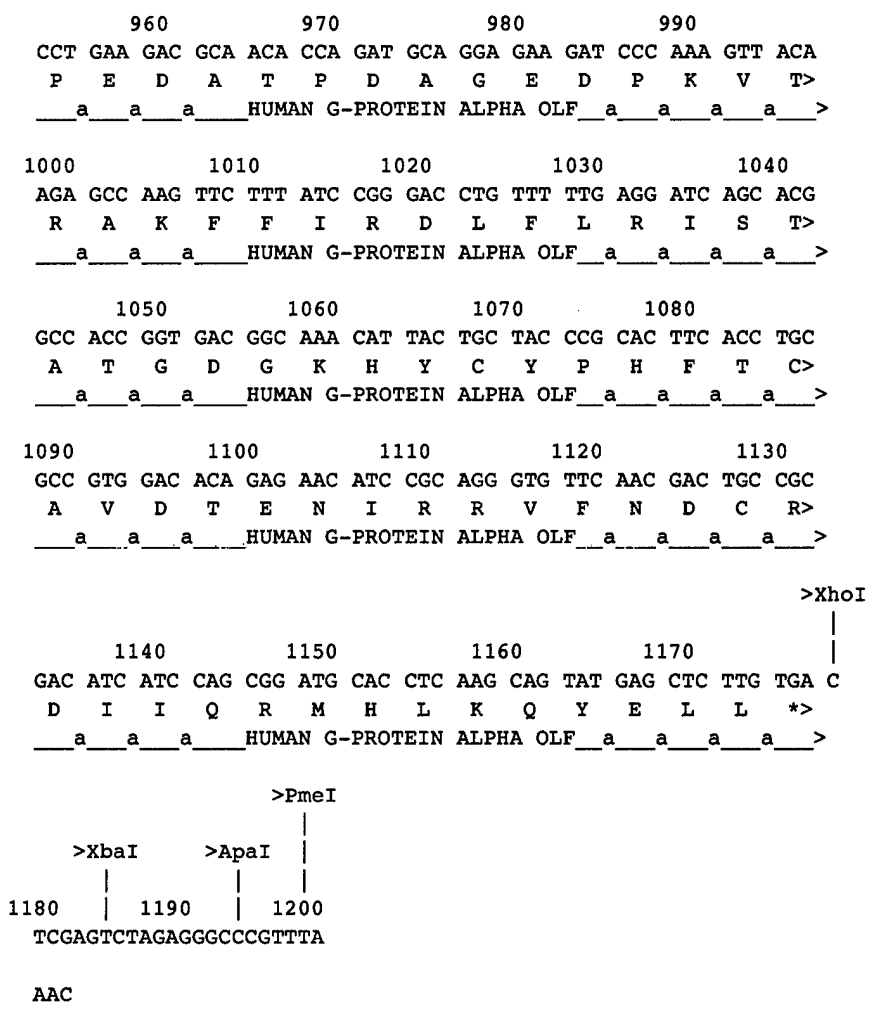
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P E D A T P D A G E D P K V T>
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1000 1010 1020 1030 1040
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HUMAN G-PROTEIN ALPHA OLF
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GCC GTG GAC ACA GAG AAC ATC CGC AGG GTG TTC AAC GAC TGC CGC
A V D T E N I R R V F N D C R>
HUMAN G-PROTEIN ALPHA OLF
>XhoI
1140 1150 1160 1170
GAC ATC ATC CAG CGG ATG CAC CTC AAG CAG TAT GAG CTC TTG TGA C
D I I Q R M H L K Q Y E L L *>
HUMAN G-PROTEIN ALPHA OLF
>PmeI
>XbaI >ApaI
1180 1190 1200
TCGAGTCTAGAGGGCCCGTTTA
AAC

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fax: (570) 882-4643

G-protein alpha olf Q214L

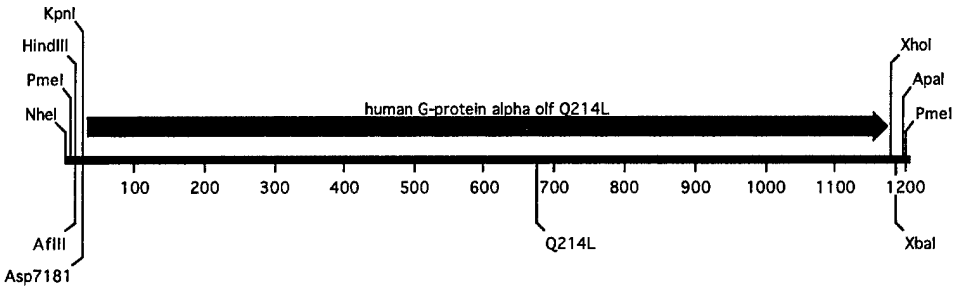
CloneID	GNA0L000C0	**Species**	human
Gene Class	G-protein alpha QL mutant	**IMAGE clone #**	
Date		**IMAGE acc. #**	
Lot	01	**Origin**	cDNA
Bacteria	JM109	**Tag**	None
Vector	pcDNA3.1+	**Tag location**	N/A
Antibiotic	Ampicillin	**Mutation**	Q214L
Promoter	CMV	**Phenotype**	CA
Insert size	1150	**Method**	Quickchange
5'RE	KpnI	**Sequenced**	Full length
3'RE	XhoI	**GB Acc. No.**	U55184

Keywords guanine nucleotide binding protein alpha human constitutively active mutant

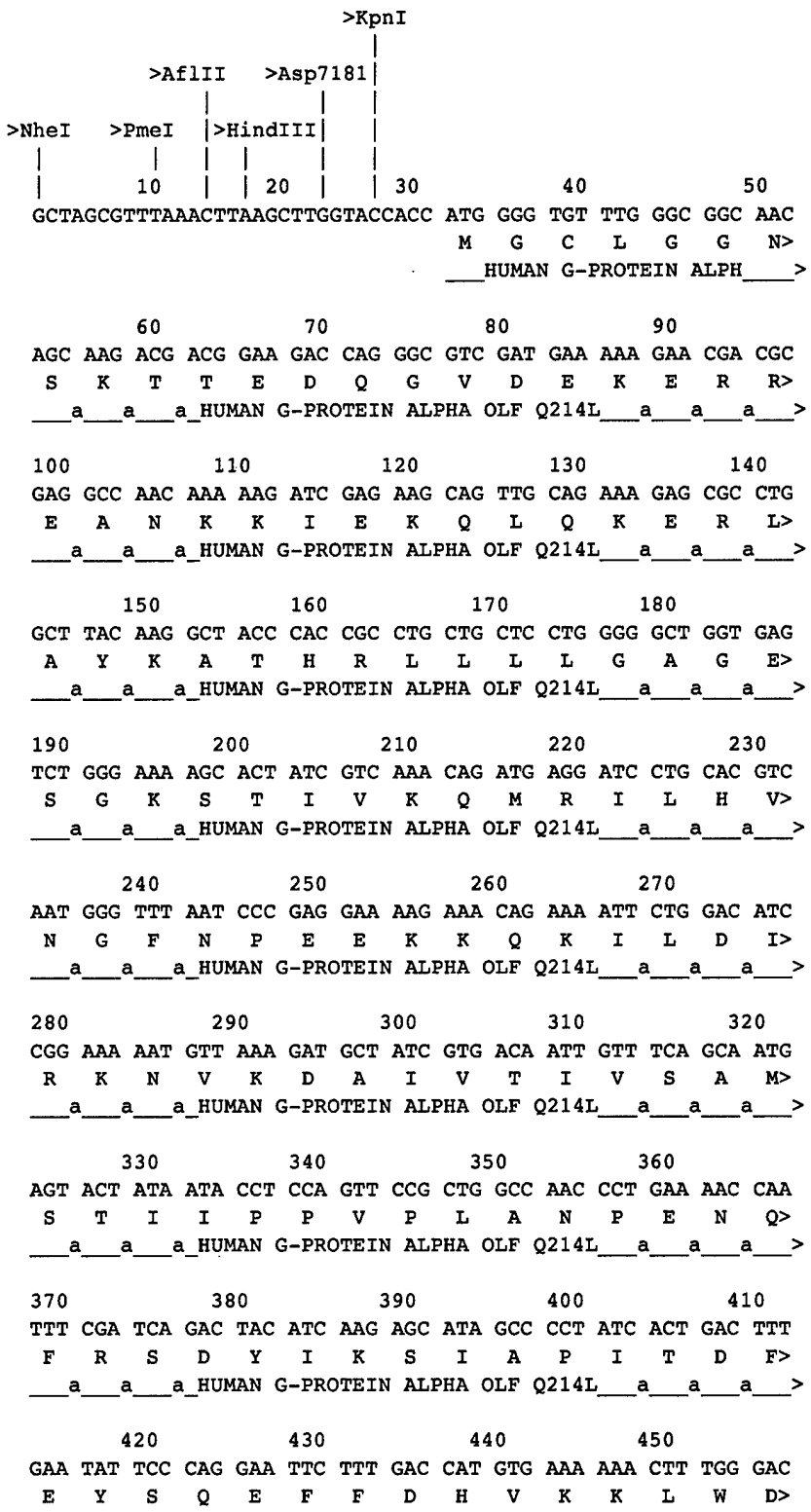
References

Notes The Q214L mutation was introduced into the human G-protein alpha olf (GNA0L00000) via the Quickchange mutagenesis kit (Stratagene). The mutation reduces GTPase activity resulting in a constitutively active phenotype. Insert size = 1150 bp.

Map



Human G-protein alpha olf Q214L



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 S   L   V   D   Y   T   P   T   D   Q   D   L   L   R   C>
___a___a___a_HUMAN G-PROTEIN ALPHA OLF Q214L___a___a___a___>

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 R   V   L   T   S   G   I   F   E   T   R   F   Q   V   D>
___a___a___a_HUMAN G-PROTEIN ALPHA OLF Q214L___a___a___a___>

                                          >Q214L
                                            |
640           650           660           670 |         680
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 K   V   N   F   H   M   F   D   V   G   G   L   R   D   E>
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       780           790           800           810
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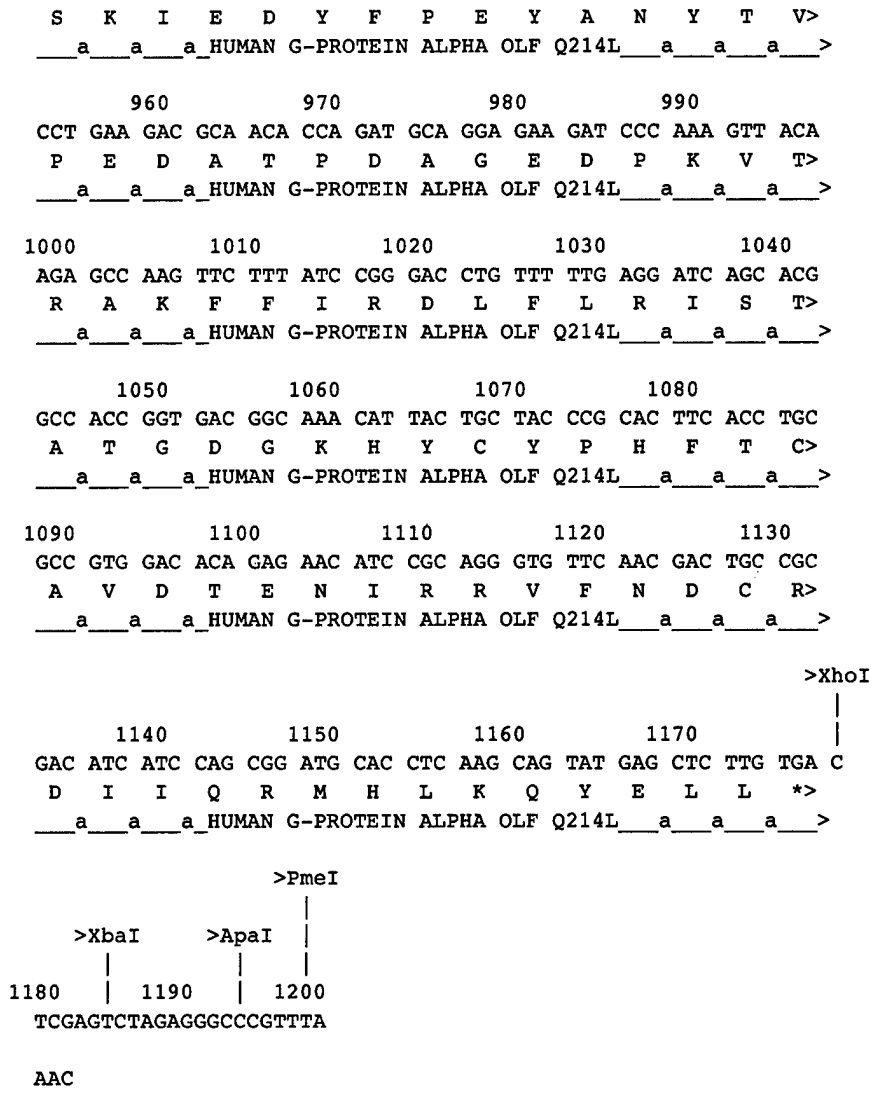
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A V D T E N I R R V F N D C R>
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>XhoI
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D I I Q R M H L K Q Y E L L *>
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>PmeI
>XbaI >ApaI
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AAC